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# Purification and characterization of a soluble calnexin from human placenta

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ABSTRACT

Calreticulin (Crt) and calnexin (Cnx) are homologous endoplasmic reticulum (ER) chaperones involved in protein folding and quality control. Crt is a soluble ER luminal Mr 46 kDa protein and Cnx is a Mr 67 kDa ER membrane protein. During purification of Crt from human placenta a soluble form of Cnx (sCnx) was consistently identified in a separate ion exchange chromatography peak. The sCnx was further purified and characterised. This showed that the protein had been cleaved after residue 472 (between Gln and Met), thus liberating it from the transmembrane and cytoplasmic parts of Cnx. The extraction and initial purification steps were carried out in the presence of protease inhibitors, thus ruling out that the cleavage was an artefact of the isolation procedure. This indicates that sCnx may have a physiological chaperone function similar to that of Crt.



## Introduction

Calnexin (Cnx)[1] and calreticulin (Crt) are important chaperones in the endoplasmic reticulum (ER) and participate in the folding and quality control of many client proteins [1–3]. The two proteins are homologous with 45% sequence identity (based on Crt) in their globular and P domains (Fig. 1; Suppl. Fig. 1). However, they differ with respect to several features: Cnx is a transmembrane protein and has a short cytoplasmic C-terminus and ER luminal N and P domains, whereas Crt is a soluble ER luminal protein with a large capacity for $Ca^{2+}$ binding in the C-terminus. Despite their similar structures and overlapping properties they seem to complement each other in the folding of other proteins, in particular the folding of MHC I [4–7]. Moreover, they seem to be able to substitute for each other in many respects [8–9]. Therefore, differences in some of their cellular functions may stem from one being a membrane protein and the other a soluble protein. However, some differences may also be related to the small differences in structure: Cnx has a longer P domain "arm" and Crt has a longer C-terminus (Fig. 1), otherwise their structures in the globular domains are essentially similar.

Previously, Crt has been isolated from human placenta and characterized extensively [10–16]. Here, the isolation and characterization of a soluble Cnx (sCnx) variant from human placenta is reported.

## Materials and methods

### Chemicals

Carbonate buffer pH 9.6 (15 mM $Na_2CO_3$, 35 mM $NaHCO_3$, 0.001% (w/v) phenolred), TTN buffer, pH 7.5 (0.025 M Tris, 0.5% (v/v) Tween 20, 0.15 M NaCl), alkaline phosphatase (AP) substrate buffer, phosphate-buffered saline (PBS) (10 mM $NaH_2PO_4/Na_2HPO_4$, pH 7.3, 0.15 M NaCl) were made in-house. Pyronin G, Tween 20, $NaH_2PO_4$, $Na_2HPO_4$, NaCl, $CH_3COOH$, $Na_2S_2O_3$, $CaCl_2$, $MgCl_2$, $(NH_4)_2SO_4$ and dimethylformamide (DMF) were from Merck (Darmstadt, Germany). Tris, BisTris, ammonium sulfate, *para*-nitrophenylphosphate (*p*NPP) substrate tablets, 5-bromo-4-chloro-3-indolylphosphate (BCIP), nitroblue tetrazolium (NBT), phenol red, Triton X-114, $NaHCO_3$, $Na_2CO_3$, $NH_4HCO_3$, glycine, urea, thiourea, guanidine (Gu) hydrochloride, trifluoroacetic acid (TFA), $CH_3CN$, glycerol, HCHO (37% (w/v), diethanolamine, AP-conjugated rabbit immunoglobulins (Igs) against mouse IgG, AP-conjugated goat Igs against rabbit IgG and endoprotease AspN (E.C. 3.4.24.33) were purchased from Sigma Aldrich (St. Louis, MO, USA). HCOOH and bovine serum albumin (BSA) were from Fluka (St. Louis, MO, USA). Protein purification equipment (Äkta Prime™), Q-Sepharose Fast Flow™, Phenyl-Sepharose Fast Flow™ and Sephacryl S-100

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[1] *Abbreviations used:* Crt, calreticulin; Cnx, calnexin; ER, endoplasmic reticulum; MHC, major histocompatibility complex; PPI, protein prolyl isomerase.

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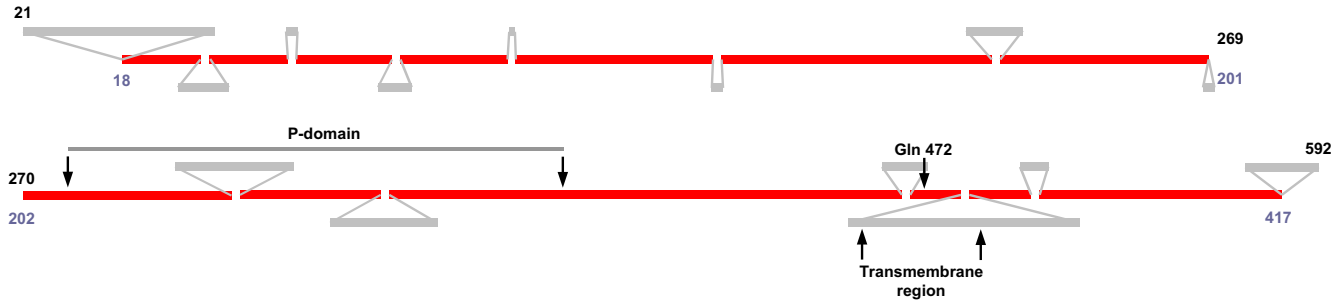


**Fig. 1.** Comparison of calnexin and calreticulin. The red line indicates the sequence of Crt and Cnx that aligns according to the alignment in Suppl. Fig. 1 with the inserts into the Crt sequence shown as grey bars. The location of the P-domain, the transmembrane region and the terminal Gln 472 of sCnx are indicated. Numbering is according to the sequence including signal sequence. Numbers above the line are for Cnx and below the line for Crt.

HR™ were from Pharmacia (Uppsala, Sweden). Sequencing grade trypsin (porcine) (E.C. 3.4.21.4) was from Promega (Madison, WI, USA). Trifluoroacetic acid (TFA) and bicinchoninic acid (BCA) protein assay kit were from Thermo Fisher Scientific (Waltham, MA, USA). MaxiSorp™ microtitre plates were from Nunc (Roskilde, Denmark). Complete protease inhibitor cocktail tablets were from Roche Diagnostics (Mannheim, Germany). Rabbit antibodies recognizing the N-terminus or C-terminus of Crt were produced as described [17]. Rabbit antibodies to ERp57 were a generous gift from Lars Ellgaard (Department of Biology, University of Copenhagen, Denmark). Rabbit antibodies recognizing the N-terminus or C-terminus of Cnx were from Calbiochem (La Jolla, CA, USA). Rabbit antibodies to ERp72 were from Abcam (Cambridge, UK). Rabbit antibodies to PDI were from Stressgen (Victoria, British Columbia, Canada). Precast Tris–glycine gels (4–20%) were from NOVEX (San Diego, CA, USA). GelCode Blue Stain Reagent and bis(succinimidyl)suberate (BS3) were from Pierce (Rockford, IL, USA). Prestained sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) molecular weight standards were from BioRad (Hercules, CA, USA). SDS and $AgNO_3$ were from BDH Chemicals (Poole, Dorset, UK). EtOH was from Kemetyl (Køge, Denmark). Poros oligo R2 microcolumns were from Applied Biosystems (Foster City, CA, USA). Ultradiafilters were from Millipore (Billerica, MA, USA). ReproSil Pur C18 AQ 3 um was from Dr. Maisch GMBH (Ammerbuch-Entringen, Germany).

### *SDS–PAGE*

SDS–PAGE was carried out according to Laemmli [18] as described by Studier [19] using precast 4–20% gels. Samples were boiled 1:2 with sample buffer (70 mM SDS, 100 mM dithiothreitol (DTT), 10% (v/v) glycerol, 0.05 M Tris pH 6.8, 0.06% (w/v) pyronin G), 15 μL was added/well and electrophoresis was carried out, using 0.024 M Tris, 0.192 M glycine, 0.1% SDS (w/v) pH 8.8 as running buffer, at 125 mA/gel. Gels were rinsed $2 \times 10$ min in water, stained with Coomassie Brilliant Blue (GelCode Blue) for 1 h and then destained in water. Silver staining of gels was done as follows: first, gels were fixed in 50% (v/v) EtOH, 12% (v/v) $CH_3COOH$ for 1 h, then they were rinsed in 30% (v/v) EtOH for $3 \times 20$ min, treated with 0.02% (w/v) $Na_2S_2O_3$ for 1 min, rinsed in water $3 \times 20$ s, stained with 0.02% (w/v) $AgNO_3$, 0.03% (w/v) HCHO for 20 min, rinsed in water $2 \times 20$ s and developed in 6% (w/v) $Na_2CO_3$, $0.4 \times 10^{-3}$% (w/v) $Na_2S_2O_3$, 0.02% (w/v) HCHO for 2–10 min. The staining was stopped with 50% (v/v) EtOH, 12% (v/v) $CH_3COOH$ for 15 min and gels finally rinsed with water.

### *Immunoblotting*

SDS–PAGE gels were sandwiched between 12 sheets of Whatman paper no 1 equilibrated in 10 times diluted electrophoresis buffer and subjected to electroblotting to nitrocellulose membranes overnight at 0.1 mA/cm$^2$ using a semi-dry electroblotting apparatus (JKA Biotech, Copenhagen, Denmark). The membranes were blocked for 1 h in 50 mM Tris, 1% (v/v) Tween 20, 0.3 M NaCl, pH 7.5 (TTN), washed three times in TTN, and then incubated with antibodies diluted 1:1000 in TTN. After 1 h incubation the membranes were washed three times in TTN, followed by incubation for 1 h with AP-conjugated rabbit Igs against mouse IgG or AP-conjugated goat Igs against rabbit IgG diluted 1:1000 in TTN. After another three washes, bound antibodies were detected by incubation with staining solution (12.5 mg 5-bromo-4-chloro-3-indolylphosphate in 0.5 ml DMF and 25 mg nitroblue tetrazolium in 0.5 ml 70% (v/v) DMF added to 75 ml 100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5).

### *Cnx and Crt purification*

Crt was purified from human placenta as previously described [11] with minor modifications (Fig. 2). A placenta was first homogenized twice with 20 mM BisTris, 1 mM $CaCl_2$, pH 7.5 in the presence of protease inhibitors (Complete tablets) followed by two rounds of homogenization with 0.5 L 20 mM BisTris, 1 mM $CaCl_2$, pH 7.5, 1% (v/v) Triton X-114. Between homogenizations, the precipitate was separated from the supernatant by 20 min of centrifu-

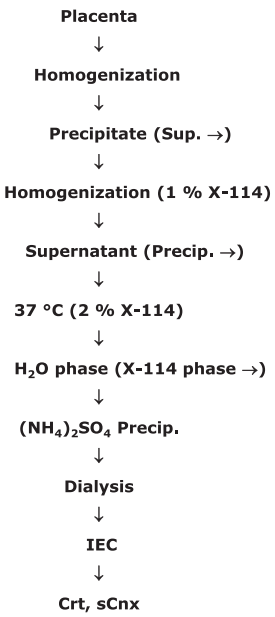


**Fig. 2.** Purification scheme for human placenta Crt. The soluble Cnx was obtained during the Q Sepharose ion exchange chromatography (IEC).

gation at 15,000*g* in a Sorvall centrifuge. The supernatants from the last two homogenizations were pooled and diluted with an equal volume of 20 mM BisTris, 1 mM $CaCl_2$, pH 7.5, 3% (v/v) Triton X-114. Subsequent temperature-dependent phase separation was performed by overnight incubation at 37 °C. The upper phase containing water-soluble proteins was isolated by aspiration and 337 g/L ammonium sulfate was added. After stirring overnight the mixture was centrifuged 1 h at 15,000*g*. The supernatant was subjected to ultradiafiltration through a 10 kDa cut-off filter against 5 volumes of 20 mM Tris, 1 mM $CaCl_2$, pH 7.5 to a final volume of 350 ml. The retentate was applied to a Q Sepharose Fast Flow ion exchange column equilibrated with 20 mM Tris, 1 mM $CaCl_2$, pH 7.5. Proteins were eluted from the column with a linearly increasing gradient of NaCl (0–0.5 M) in 20 mM Tris, 1 mM $CaCl_2$, pH 7.5. (Flow rate: 1 mL/min, 6 column volumes in all). Crt-containing fractions were identified by SDS–PAGE and enzyme-linked immunosorbent assay (ELISA) using antibodies directed against Crt's C-terminus [17]. Similarly, Cnx-, ERp57-, ERp72- and protein disulphide isomerase (PDI)–containing fractions were located by SDS–PAGE and ELISA.

For some purposes, Crt was further purified by size exclusion chromatography on a Sephacryl S-100 column (16 cm × 80 cm) equilibrated and eluted with 20 mM Tris, pH 7.5 (Flow rate: 1 mL/min).

Cnx was further separated from ERp72 by hydrophobic interaction chromatography on a Phenyl-Sepharose 6 Fast Flow column equilibrated in 50 mM sodium phosphate, 1.7 M $(NH_4)_2SO_4$, pH 7.0 and eluted with a decreasing gradient of ammonium sulfate (1.7 M) in 50 mM sodium phosphate, pH 7.0.

### *ELISA*

Fractions were coated onto the surface of the wells of microtitre plates (MaxiSorp), using 0.05 M sodium carbonate, pH 9.6 as coating buffer (dilution 1:50). Coating was carried out overnight at 4 °C using 100 μL/well. After coating, the plates were washed three times for 1 min in washing buffer (TTN; 50 mM Tris, 0.15 M NaCl, 1% (w/v) BSA, 1% (v/v) Tween 20; or PBS, pH 7.3, 0.5% (v/v) Tween 20), blocked for 1 h in washing buffer, followed by 1 h incubation at room temperature with antibodies diluted 1:1000 in incubation buffer (the same as washing buffer). The plates were washed 3 times 1 min in washing buffer, incubated 1 h with AP-conjugated goat Igs against rabbit IgG, or AP-conjugated rabbit Igs against mouse IgG 1:1000 in incubation buffer, followed by three washes. Finally, bound antibodies were quantified using *p*-NPP (1 mg/ml) in AP substrate buffer (1 M diethanolamine, 0.5 mM $MgCl_2$, pH 9.8). The absorbance was read at 405 nm with background subtraction at 690 nm.

### *Mass spectrometry (MS)*

#### *Intact mass analysis*

A Cnx sample was denatured by 10 mM DTT in 6 M GuHCl, 100 mM Tris buffer, pH 8.3 for 3 h at 37 °C. The buffer was subsequently exchanged by 50% (v/v) $CH_3CN$, 0.1% (v/v) formic acid and injected into the nano source via a syringe pump (direct infusion) at a flow rate of 20 μL/h. The nano source was coupled to a Waters Premier QTOF (Waters Micromass, Manchester, UK).

The mass spectrometric conditions were the following: positive ion mode; API collision gas: on; capillary: 2.3 kv; sampling cone: 3.5; extraction cone: 2.5; ion guide: 2.9; temperature: source: 80 °C; nano flow: 0.2 bar.

#### *Bottom-up analysis*

Three times 5 μg Cnx was lyophilized and re-dissolved in 6 M urea, 2 M thiourea, reduced and alkylated. The samples were diluted by 10 vol 50 mM $NH_4HCO_3$ and digested by 2% (w/w) trypsin, and 2% (w/w) Asp-N overnight at 37 °C separately. The peptide mixtures were desalted using Poros Oligo R2 RP microcolumns.

The nanoLC-MS/MS was performed by a Proxeon 1100 easy-nLC system (Thermo Fisher Scientific/Proxeon Biosystems, Odense, Denmark). The peptides were loaded onto a 100 μm ID, 18 cm RP capillary column (packed in house with ReproSil Pur $C_{18}$ AQ 3 μm sorbent) in buffer A (0.1% (v/v) formic acid, 5% (v/v) $CH_3CN$). The peptides were eluted using a 50 min gradient from 0 to 34% B-buffer (95% (v/v) $CH_3CN$, 0.1% (v/v) formic acid) at 350 nL/min flow rate and via nanoelectrospray introduced into an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). A full MS scan in the mass area of 300–1800 m/z was performed in the Orbitrap with a resolution of 30.000 FWHM and a target value of $1^*10(6)$ ions. For each full scan the top 5 intense ions (>+1 charge state) were selected for collision-induced dissociation (CID). Parameters for acquiring CID were as follows: activation time, 15 ms; normalized energy, 35; Q-activation, 0.25; dynamic exclusion, enable with repeat count 1; exclusion duration, 30 s.

#### *Chemical cross-linking*

50 μg Cnx interacted with 1.5 mg BS3 for 1 h and the reaction was quenched by 100 mM $NH_4HCO_3$. The sample was reduced, alkylated and digested by 2% (w/w) trypsin. The cross-linked peptides were separated by strong cation exchange (SCX) chromatography using a polySULFOETHYL A, $150^*1$ mm, 5 μm particles, 300 Å pore size column (Poly LC Inc, Columbia, MD, USA). The buffer system was A: 0.1% (v/v) TFA, 30% (v/v) $CH_3CN$, B: A + 0.5 M NaCl and the gradient was: 0% B (10 min); 0–40% B (30 min); 40–90% B (5 min); 100% B (5 min), at a flow rate of 50 μL/min. The separation was performed on an Agilent 1200 HPLC (Agilent Technologies, Santa Clara, CA, USA). All peaks were collected and divided into 4 fractions. Each fraction was desalted using Poros Oligo R2 RP microcolumns.

Samples were run by nano LC–MS/MS like for bottom-up analysis, except that the selected ions were selected for high energy collision dissociation (HCD) and detected at a resolution of 7500 FWHM. The settings for the HCD were as follows: threshold for ion selection, 15.000; target value of ions used for HCD $2^*10(5)$; activation time, 10 ms; isolation width, 2.5; normalized collision energy, 42; repeat count, 1; repeat duration, 30; exclusion duration, 45.

#### *Data analysis*

Bottom-up analysis was performed using X!Tandem (2010.01.01.4) through the GPMAW software version 9.21 (Lighthouse Data, Odense, Denmark). Search parameters were: Parent error 8 ppm; fragment error 0.5 Da, max. e-value 0.001; partial modifications: oxidation of Met; fixed modifications: carbamidomethylation of Cys; semi cleavage enabled (Suppl. Fig. 3). Identification of cross-linked peptides was done using CrossWork version 0.105 [20].

## Results

In Fig. 2, a purification scheme used for Crt purification is shown and Table 1 summarizes typical results from two purifications. Initially, a human placenta was cut into small pieces and homogenized in a food processor using 0.5 L 20 mM BisTris, 1 mM $CaCl_2$, pH 7.5 as buffer. After centrifugation, the precipitate was re-homogenized with an equal volume of BisTris buffer. After another centrifugation, the precipitate was homogenized in the same way twice with 0.5 L 20 mM BisTris, 1 mM $CaCl_2$, pH 7.5, 1% (v/v) Triton X-114 as buffer. The combined supernatants from this detergent

**Table 1**
Purification yields of Crt (A, B) and sCnx (C) from human placenta. ND: not determined. NA: not applicable.

| Step | Volume mL | Protein (P) mg[a] | Crt, Cnx mg[b,e] | Purity (Crt, Cnx/P) | Protein A280[c,f] | Yield (%) | Purification factor |
|---|---|---|---|---|---|---|---|
| *A (Crt)* | | | | | | | |
| Sup. after Homog. | 1650 | 9900 | 25 | $3\ 10^{-3}$ | ND | 100 | 1 |
| Sup. after Precip. | 2660 | 1596 | 21 | $13\ 10^{-3}$ | ND | 84 | 4.3 |
| Dialysis | 360 | 1656 | 13 | $8\ 10^{-3}$ | ND | 52 | 2.7 |
| Q-Sepharose eluate | 62 | 19 | 15 | 0.8–0.9 | 16 | 60 | 300 |
| Diafiltration | 12 | 13 | 12 | 0.9–1.0 | 12 | 48 | 333[d] |
| *B (Crt)* | | | | | | | |
| Sup. after Homog. | 1730 | 12,456 | 47 | $4\ 10^{-3}$ | ND | 100 | 1 |
| Sup. after Precipitation | 2825 | 2260 | 31 | $13\ 10^{-3}$ | ND | 66 | 3.3 |
| Dialysis | 340 | 1632 | 22 | $13\ 10^{-3}$ | ND | 47 | 3.3 |
| Q-Sepharose eluate | 54 | 18 | 20 | 1.0–1.1 | 20 | 42 | 250[d] |
| Diafiltration | 15 | 17 | 16 | 0.9 | 17 | 34 | 225 |
| Step | Volume mL | Protein (P) mg[a] | Crt mg[e] | Purity (Crt/P) | Protein A280[f] | Yield (%) | Purification factor |
| *C (Cnx)* | | | | | | | |
| Sup. after Homog. | 1730 | 12,456 | 155 | $12\ 10^{-3}$ | ND | 100 | 1 |
| Sup. after Precipitation | 2825 | 2260 | 11 | $5\ 10^{-3}$ | ND | 7 | 0.4 |
| Dialysis | 340 | 1632 | 26 | $15\ 10^{-3}$ | ND | 17 | 1.3 |
| Q-Sepharose eluate | 70 | ND | 3 | NA | ND | 2 | NA |
| Diafiltration | 6 | ND | 3 | NA | ND | 2 | NA |

[a] BCA assay.
[b] ELISA.
[c] $E_{280}$ (Crt) = 2.13.
[d] Theoretical limit.
[e] SDS–PAGE scanning.
[f] $E_{280}$ (Cnx) = 1.

extraction were diluted with an equal volume of the same buffer with 3% (v/v) Triton X-114 to reach a final detergent concentration of 2% (v/v) and incubated af 37 °C to induce temperature-dependent phase separation. The water phase, containing the soluble ER proteins, was subjected to ammonium sulfate precipitation and the supernatant from this, containing very soluble ER proteins and some contaminating proteins was further purified.

Fig. 3 shows the resulting ion exchange chromatogram of proteins from the supernatant of the ammonium sulphate precipitation, when loaded on a Q-Sepharose column and eluted by a linearly increasing salt gradient. Fractions were monitored by ELISA (Fig. 3) and selected fractions analyzed by SDS–PAGE (Fig. 4). Major bands in the SDS–PAGE gels were identified by MS and the elution profiles of dominating proteins are shown in Fig. 3. Crt elutes at ~0.45 M NaCl in almost pure form as the last and major peak. Interestingly, ERp57 elutes well before and completely separated from Crt at ~0.3 M NaCl. The prominent peak eluting at 0.40 M NaCl just before the Crt peak turned out to be a mixture of ERp72 and Cnx. The presence of Cnx was unexpected, since it is a membrane protein. However, it was reproducibly observed in the same amount relative to Crt, approximately 3 mg Cnx and 15 mg Crt per placenta (Table 1). The yield of Crt relative to the starting homogenate was approximately 40% and the purity was close to 100%, a conclusion supported by SDS–PAGE and LC–MS analysis. The yield relative to the total amount of Crt in a placenta is not known, but from SDS–PAGE and immunoblotting analyses of the extracts it was estimated to be approximately 20%, that is, about half of the Crt present in a placenta is recovered in the detergent extract, and about half is lost in the first buffer extract. The yield of sCnx relative to the total amount of Cnx in a placenta can-

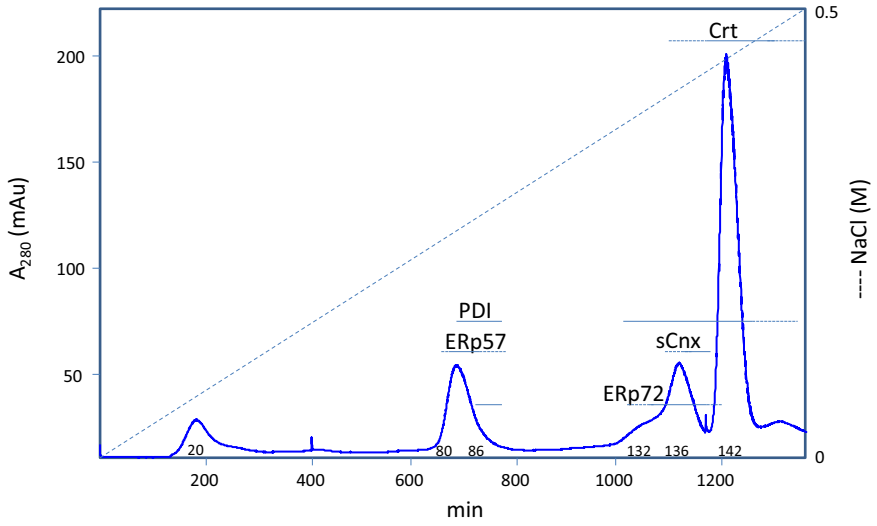


**Fig. 3.** Chromatogram from Q-Sepharose chromatography of human placenta ER extract. Elution profiles of major protein constituents are indicated and underlining shows relative ELISA absorbance values (absorbance value divided by maximum value for the specific antibody) (—: 0.1–0.3, ——: 0.3–1). The locations of fractions analysed by SDS–PAGE are shown by small numbers. The chromatography has been performed 10 times with identical results.

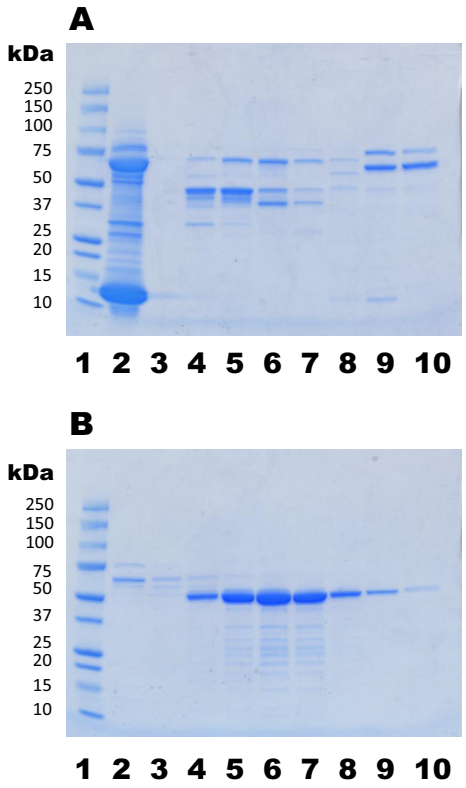


**Fig. 4.** SDS–PAGE analysis of selected fractions from Fig. 3. The gel was stained with Coomassie Brilliant Blue and the contents of the lanes are as follows: A. 1. Molecular weight standard, 2. ER extract, 3, Fr. 20, 4. Fr. 80, 5. Fr. 83, 6. Fr. 86, 7. Fr. 91, 8. Fr. 125, 9. Fr. 132, 10. Fr. 134. B. 1. Molecular weight standard, 2. Fr. 136, 3. Fr. 138, 4. Fr. 140, 5. Fr. 141, 6. Fr. 142, 7. Fr. 144, 8. Fr. 146, 9. Fr. 147, 10. Fr. 148.

not be estimated. The purity of the sCnx after the ion exchange chromatography was approximately 60%. The major other protein present was ERp72, identified by MS [19]. The two proteins behaved very similarly during chromatography, but could be separated by hydrophobic interaction chromatography (HIC) (Fig. 5).

Characterization of the soluble Cnx by MS showed that it was terminated after residue 472 (Gln) (Fig. 1, Suppl. Fig. 3) as shown by bottom-up analysis using trypsin and Asp-N. This showed a coverage of 95.6% with no modifications observed (Suppl. Fig. 3). The intact molecular mass was measured to be 51.412 Da (theoretical mass of Cnx 21–472 is 51.409 Da; Suppl. Fig. 2), showing that the sCnx had an intact N-terminus starting at residue 21 and did not contain posttranslational modifications other than disulphide bonds. Thus, the soluble Cnx corresponds to the globular and P domains of Crt (Fig 1). This explains its solubility, since the transmembrane part had been cleaved off together with the cytoplasmic C-terminus by a specific but yet unknown mechanism. It should be noted that the whole purification starts with homogenization in the presence of a protease inhibitor cocktail, and that the Crt elution profile shows no signs of proteolytic degradation and neither do ERp57 or ERp72 [20] (Fig. 6).

The purification scheme (Fig. 2) was changed systematically in several ways to investigate the influence of the different steps on the elution profile of the major proteins (i.e., omission of the ammonium sulfate precipitation and omission of phase separation and ammonium sulfate precipitation). However, this did not change the order of elution of the proteins and ERp57 eluted well separated from Crt under all conditions, with and without $Ca^{2+}$ added (results not shown).

Chemical cross-linking of the soluble Cnx was carried out in three different experiments, and positive identification was only accepted if present in at least two experiments. In this way 17 cross-links were identified (Table 2) and the corresponding distances were measured in the only known structure of calnexin, the dog calnexin (PDB accession number 1JHN).

Cross-linked peptides from the contaminating ERp72 were also observed. These conformed to the ones previous observed [20].

## Discussion

Purification of Crt from human placenta was slightly improved compared to previous procedures by inclusion of $Ca^{2+}$ in extraction and chromatography buffers. This makes it possible to purify Crt with only a single chromatographic step (ion exchange chromatography), whereas a size exclusion chromatography step was usually necessary to obtain pure Crt without addition of $CaCl_2$. As shown in Table 1, this reproducibly yielded about 15 mg of highly pure Crt from a placenta. Crt behaved as a soluble non-associated protein

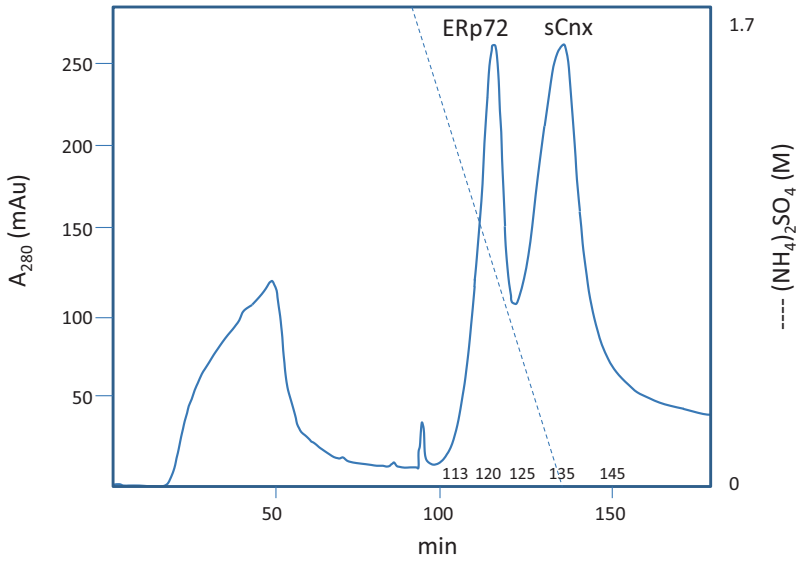


**Fig. 5.** Hydrophobic interaction chromatography of sCnx-containing peak from Fig. 3. Elution profiles of major protein constituents are indicated and the locations of fractions analysed by SDS–PAGE are shown by small numbers. The chromatography has been performed 3 times with identical results.

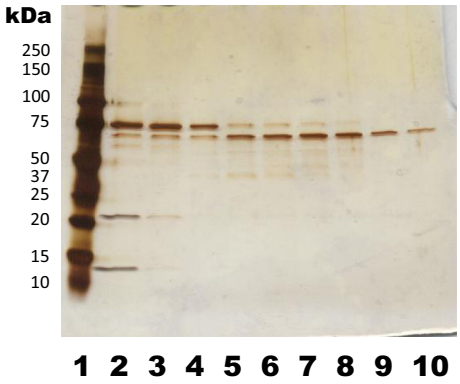


**Fig. 6.** SDS–PAGE analysis of selected fractions from Fig. 5. The gel was silver-stained and the contents of the lanes are as follows: 1. Molecular weight standard, 2. Fr. 113, 3. Fr. 117, 4. Fr. 120, 5. Fr. 125, 6. Fr. 130, 7. Fr. 135, 8. Fr. 140, 9. Fr. 145, 10. Fr. 150.

**Table 2**
Cross-links observed in sCnx by cross-linking with BS3, digestion with trypsin, analysis by LC–MS/MS and identification by CrossWork. Only cross-linked peptides observed in at least two out of three separate experiments are shown. Numbering is based on the active human calnexin sequence (UniProt P27824). A few residues (70, 79 and 438) are not part of the pdb structure (1JHN) and the distances listed in brackets are to residues ultimate ([]) or penultimate ([[]]) to the linked residue.

| Fraction number | Crosslink | Number of fragment ions | Alpha carbon distance (Å) |
|---|---|---|---|
| 4 | 67–70 | 17 | [6.3] |
| 4, 3 | 150–187 | 15 | 13.8 |
| 3, 2, 1 | 67–83 | 16 | 12.1 |
| 4 | 70–107 | 22 | [10.9] |
| 4 | 83–107 | 22 | 8.7 |
| 4 | 107–213 | 18 | 21.1 |
| 4 | 67–107 | 16 | 12.4 |
| 4 | 187–197 | 15 | 9.9 |
| 4, 3 | 98–107 | 15 | 21.7 |
| 4 | 107–438 | 14 | [30.9] |
| 4 | 79–107 | 11 | [[24.7]] |
| 4, 3, 2 | 69–83 | 23 | 14.6 |
| 3 | 187–438 | 14 | [40.1] |
| 3 | 83–213 | 13 | 21.1 |
| 3 | 70–83 | 19 | [14.6] |
| 3 | 93–213 | 14 | 13.4 |
| 3, 2 | 98–213 | 10 | 16.1 |

under all conditions, with and without $Ca^{2+}$ present. This indicates that human placenta Crt does not form stable complexes with other proteins, including ERp57 and PPI. During this work, a soluble Cnx was identified, isolated and characterized as a "side product" of Crt purification. The sCnx behaved very similar to Crt and eluted as a separate peak together with mainly ERp72 just before the Crt peak (Fig. 3). Further purification to homogeneity of sCnx proved somewhat difficult as it behaved similar to ERp72 in many respects. However, partial separation of the two was achieved by hydrophobic interaction chromatography on Phenyl-Sepharose. The yield of sCnx from a placenta was very reproducible, approximately 3 mg compared to 15 mg Crt per placenta. Thus, sCnx amounted to approximately 20% of the Crt amount, which was quite significant, considering that Cnx is a membrane protein, and would not be expected to co-purify with Crt since the extraction was carried out in the presence of protease inhibitors, and since no signs of Crt proteolysis were seen.

Previously, a Crt form lacking the C-terminal hexapeptide has been observed in Crt preparations also obtained after extraction in the presence of protease inhibitor cocktails [11]. This form arose by cleavage between Gly and Gln. Characterization of the soluble Cnx showed that it had been cleaved between Gln 472 and Met 473, thus liberating if from the ER membrane. No signs of heterogeneity in the cleavage site were found, neither by chromatography nor by mass spectrometry, thus suggesting cleavage by a rather specific protease. Cathepsin P, a member of the group of placentally expressed cathepsins [21], has been reported to be capable of limited processing of calreticulin from the C-terminus [22], which obviously removes the KDEL sequon. Other placentally expressed proteases are cathepsin L and B [21,23], metalloproteases [24] and caspases [25]. Crt has been found to be a substrate for at least one caspase [26] and the cleavage sites defined by the Crt form lacking the C-terminal hexapeptide [11] and the sCnx described here could indicate processing by a caspase or caspase-like enzyme. Alternatively, some kind of chemical cleavage or autocleavage could have occurred.

In order to verify the structure of the soluble Cnx, three samples were cross-linked with BS3, digested with trypsin and analysed by mass spectrometry following previous procedures [20]. Of the 17 observed cross-links, 11 were defined in the only released Cnx structure (PDB 1JHN) and were all well within the size limits of BS3 and two times lysine (~25 Å). Four linkages were observed to a flexible loop region (residues 70–80), but the distances still conform to the expected limits. Two linkages were observed from residue 438 to residue 128 and 208, respectively, which are highly unlikely based on the published structure due to a length of 30.9 Å and 40.1 Å. Residues 128 and 208 are both located on the opposite surface of Cnx relative to the C-terminal residue 437 of 1JHN. This indicates that while the majority of the protein has the same conformation as 1JHN, the C-terminal end of sCnx may be in a different conformation that brings residue 428 close to the opposite surface of sCnx.

The existence of a soluble form of Cnx in substantial amounts raises the question of a possible physiologic function of this sCnx. Cnx and Crt can substitute for each other with regard to several chaperone activities and appear to have overlapping substrate specificities [8,9,27,28]. Therefore, a soluble Cnx may simply carry out some of the functions also provided by Crt. Crt has also been reported to function as a signalling molecule, mainly in relation to apoptosis, where it appears on the cell surface as one of the first detectable molecules [29,30]. A similar function may be performed by sCnx. Indeed, Cnx has been reported to be exposed on the surface upon apoptosis [31].

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.pep.2013.09.006.

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